1689

PHOSPHATE HEAT TREATMENT OF MILK TO PREVENT BACTERIOPHAGE PROLIFERATION IN LACTIC CULTURES

R. E. HARGROVE, F. E. McDONOUGH, AND R. P. TITTSLER

Dairy Products Laboratory, Eastern Utilization Research and Development Division, USDA
Washington, D. C.

SUMMARY

The effects of various prosphate salts on bacteriophage proliferation in milk cultures and the binding of calcium in milk were studied, with the aim of developing a practical and reliable method for preventing bacteriophage development in lactic starter cultures. The kind and concentration of phosphate, pH, and heat treatment had a marked effect on phage inhibition, and the free calcium content of milk. Of the group of phosphate salts tested individually in milk, orthophosphates showed the greatest degree of bacteriophage inhibition. Most of the phage types tested were suppressed by 2% orthophosphate (salt), but the most resistant types required 3%. Heating the milk after adding the phosphate was essential to sufficiently bind most of the free calcium. Usually, the free calcium content of the treated milk ranged from 10 to 30 p.p.m. The phosphated milk became more effective as the pH of the phosphate buffer and milk was increased from pH 6.4 to 7.0. The best combination for phage inhibition, minimum milk precipitation, and economy was obtained when milk was heated with 1.7% orthophosphate salt, pH 6.6, followed by the addition of 0.3% pyrophosphate. Thirteen different lactic strains grossly contaminated with their respective phages were freed of phage within three to four subcultures in phosphated milk. In most instances, the activity of the cultures in phosphated milk was as great as or greater than in the controls.

The requirement of divalent metals, especially calcium, for the proliferation of bacteriophage (phage), has been established (1, 4, 8). Particular attention has been given to the calcium requirements of lactic phages, because of the economic importance of starter failures in making cheese and other cultured dairy products (4, 9). Several attempts have been made to develop a medium which would prevent or suppress the proliferation of phage in lactic cultures. Potter and Nelson (10) developed a calcium-deficient, semichemically defined medium. Doull and Meanwell (5) proposed a sodium citrate–containing medium. More recently, Reiter (11) developed a calcium-deficient milk medium designated as a phage resistant medium, or simply as P.R.M.

Because of an apparent inhibitory effect of P.R.M. on some strains of lactic bacteria (3), and the lack of a suitable milk medium for propagating phage-free starters, this laboratory has investigated the feasibility of adding phosphate to lactic starter-milk with the aim of chemically binding the essential calcium and thereby preventing phage proliferation in lactic cultures.

Preliminary results (2, 7) showed that the addition of 2% orthophosphate to milk, combined with a mild heat treatment, prevented the development of almost all types of lactic phages. Since then, the effects of kind and concentration of phosphate, pH, heat treatment, and percentage of milk solids on the binding of calcium, the inhibition and elimination of phage proliferation, and the activity of lactic cultures have been determined in greater detail. Typical data from the accumulated results are summarized here.

MATERIALS AND GENERAL METHODS

Bacteria and phages. Thirty-four strains of lactic streptococci and homologous phages were either isolated from cheese and cultured buttermilk or obtained from other laboratories.

Stock suspensions of phages were prepared from lysed homologous lactic strains grown in sterilized skimmilk. The curd was precipitated with 85% lactic acid and removed by filtering. The whey was neutralized and passed through a Seitz ST-1 filter.

Phage activity. The phage-host relationship of each of the 34 lactic strains was determined by spotting serial dilutions of each phage on agar plates seeded with an individual bacterial strain. Thirteen different types of phage were indicated and each type was employed in the study.

Quantitative phage determinations were made by a two-layer agar plaque technique. In addition to plaque counts, the phage titers were frequently determined with the dilution end point method in sterile skimmilk.

In most instances, the effectiveness of a particular phosphate treatment was determined by inoculating the milk wth fixed volumes (1%) of culture and homologous phage and measurng the phage titers after each of several passages in the phosphated milk.

Phosphate solutions. For most of the work, concentrated stock solutions (50% w/v) orthophosphate buffer, pH 6.55, were prepared by dissolving 30 g. of KH_2PO_4 and 20 g. Na_2HPO_4 in distilled water with the aid of heat and bringing the volume to 100 ml. An appropriate volume of stock solution was added to milk to yield the desired concentration of phosphate (w/v). A buffer concentrate (50% w/v) containing equal amounts of K_2HPO_4 and $NaH_2PO_4 \cdot H_2O$, pH 6.63, was added to milk to obtain a 3% concentration. Varied ratios of primary and secondary phosphates were used in preparing concentrated solutions (50-60% w/v) having different pH levels. Concentrated stock solutions of pyrophosphate contained (50% w/v) tetrapotassium pyrophosphate.

Calcium analysis. The indirect titration method of Yalman *et al.* (12) was used in determining the ionizable calcium content of the milk. Analyses were made on the phosphate-treated and nontreated skimmilks, and on their supernatants following centrifugation for 90 min. at 40,000 r.p.m. A 25-ml. sample was evaporated to dryness on a steam bath, the solids determined, and then reduced to ash overnight in a muffle furnace at 650° C. The calcium values for the supernatants are reported as unbound calcium in parts per million.

Bacterial activity. The activity of lactic bacteria following growth in phosphate-containing milk was determined by a 6-hr. activity test. Inoculation was at the rate of 1% and incubation was at 88° F. The effect of the phosphate milk on the growth of *Leuconostoc* strains was determined by plate count.

The number of *Leuconostoc* cells in mixed lactic cultures was determined with a selective plating medium developed in this laboratory (to be published).

EXPERIMENTAL PROCEDURES AND RESULTS

Effect of phosphate concentration. Stock (50% w/v) orthophosphate buffer, pH 6.55, was added to fresh skimmilk to yield 1.0, 1.5, 2.0, and 3% of phosphate by weight. The phosphated milks and a control were heated at 100° C. for 15 min., cooled, and individual tubes inoculated with 1% of *Streptococcus cremoris,* Strains R-1, C-13, and ML-1, and their homologous phages. The phage titer in each culture was determined at inoculation, after incubation for 18 hr. at 72° F., and after each of three subcultures in the respective phosphated milk.

The data in Table 1 show that the phage for C-13 was suppressed by 1% phosphate, R-1 by 1.5 to 2%, and ML-1 by 3.0%. Other results showed that 2% phosphate was sufficient to prevent the proliferation of most types of lactic phage. However, two types, both specific for Lactic Strains C-1 and C-10, required as much as 3%.

TABLE 1

Effect of orthophosphate concentration on phage proliferation

Phosphate	Unbound calcium	Lactic strain	Phage titer (No/ml) Inoculum	18-hr. culture	Subculture 1st	Subculture 2nd	Subculture 3rd
(%)	*(p.p.m.)*						
None	228	R-1	22×10^6	92×10^7	*	*	*
1.0	66	R-1	22×10^6	76×10^7	*	*	*
1.5	35	R-1	22×10^6	91×10^4	12×10^4	20×10^4	810
2.0	30	R-1	22×10^6	115×10^4	48×10^2	20	0
2.5	24	R-1	22×10^6	180×10^4	8×10^3	80	0
3.0	16	R-1	22×10^6	145×10^4	8×10^3	10	0
None	228	C-13	11×10^6	132×10^7	*	*	*
1.0	66	C-13	11×10^6	1×10^5	4×10^3	12	0
1.5	35	C-13	11×10^6	13×10^4	630	0	0
2.0	30	C-13	11×10^6	23×10^4	140	0	0
2.5	24	C-13	11×10^6	34×10^4	100	0	0
3.0	16	C-13	11×10^6	63×10^4	380	0	0
None	228	ML-1	48×10^5	136×10^7	*	*	*
1.0	66	ML-1	48×10^5	24×10^8	*	*	*
1.5	35	ML-1	48×10^5	20×10^8	*	*	*
2.0	30	ML-1	48×10^5	31×10^8	*	*	*
2.5	24	ML-1	48×10^5	57×10^4	30×10^5	18×10^5	12×10^6
3.0	16	ML-1	48×10^5	8×10^3	20×10^2	40	0

* Culture lysed, no subculture.

The content of free calcium in phosphated milk decreased greatly with increased concentration of phosphate.

Effect of pH. Orthophosphate buffers having pH values in the range of 6.25 to 7.4 were added at a 2% by weight level to fresh skimmilk and the treated milks were heated at 100° C. for 15 min. The resulting milks, representing pH values from 6.3 to 7.1, were used in testing phage strains for *S. cremoris* KH and ML-1. The phage titers at the time of inoculations and after each of three subcultures in the respective media are shown in Table 2. The data clearly show

TABLE 2

Effect of pH of phosphated milk on phage proliferation

pH of buffer	pH of milk	Calcium content	Lactic strain	Phage titer (No/ml)				
				Inoculum	18-hr. culture	Subculture 1st	2nd	3rd
		(p.p.m.)						
6.25	6.3	44	KH	12×10^5	17×10^8	*	*	*
6.32	6.35	34	KH	12×10^5	46×10^8	*	*	*
6.4	6.42	33	KH	12×10^5	20×10^3	128×10^2	5×10^3	0
6.55	6.5	24	KH	12×10^5	21×10^3	15×10^3	0	0
6.88	6.68	21	KH	12×10^5	49×10^2	100	0	0
7.00	6.76	19	KH	178×10^5	11×10^3	20	0	0
7.10	6.88	20	KH	178×10^5	9×10^3	50	0	0
7.2	7.0	16	KH	178×10^5	2×10^3	30	0	0
7.4	7.1	13	KH	178×10^5	3×10^3	30	0	0
6.55	6.5	24	ML-1	26×10^4	58×10^7	$>10^7$	*	*
6.88	6.68	21	ML-1	26×10^4	36×10^3	84×10^6	*	*
7.00	6.76	19	ML-1	26×10^4	17×10^3	41×10^3	63×10^4	27×10^3
7.10	6.88	20	ML-1	26×10^4	23×10^3	24×10^2	49×10^2	110
7.2	7.0	16	ML-1	26×10^4	118×10^2	280	0	0
7.4	7.1	13	ML-1	26×10^4	94×10^2	30	0	0

* Culture lysed, no subculture.

a marked influence of pH on phage inhibition and elimination, the higher pH levels being much more effective. A pH of 6.4 to 6.55 in the milk was required to free Strain KH. Tests with other phage types showed that pH 6.55 was sufficient to eliminate most phage strains from the lactic cultures. However, with 2% phosphate, a pH of a least 7.0 was required to inhibit phage for Strains ML-1 and C-10. The calcium levels in the treated milks decreased slightly as the pH of the buffer was increased.

Effect of heating. Sterile stock buffer, pH 6.55, was added aseptically to flasks of sterile (251° F.—15 min.) and pasteurized (143° F.—30 min.) skimmilk to provide a 2% level of phosphate salt. Individual flasks of pasteurized phosphated milks were heated at 100° C. for 15, 30, 60, and 90 min., respectively. Individual flasks of sterile phosphated milks were heated for 15 min. at temperatures ranging from 70 to 100° C.

Another series of pasteurized and sterile milks was treated with pH 7.28 buffer at the 2% level and given similar heat treatments. Milks with pH 6.55 buffer were inoculated with Lactic Strain R-1 and its phage, and milks with pH 7.28 buffer were inoculated with Strain ML-1 and its phage. The effects of heating phosphate-containing milks are shown in Table 3. A minimum heat treatment of 100° C. for 30 min. was required when pasteurized milk was the starting medium, but much less heat was needed with sterile milk. A temperature of 80° C. for 15 min. was sufficient with R-1 phage; however, 95 to 100° C. was required with other strains. Heating had a marked effect on the chemical binding of the calcium by the phosphate. Increases in temperature and period of exposure usually resulted in lesser amounts of free calcium in the milks.

Effect of milk solids. Stock buffer, pH 6.55, was added at the 2% phosphate level to raw and sterile skimmilks and to raw and sterile skimmilks freshly reconstituted from NFDM powder to levels of 8, 10, 12, and 14% solids.

TABLE 3

Effect of heating phosphated milk on phage proliferation

Type of milk	Heat treatment	Calcium content	Lactic strain	Phage titer (No/ml)				
				Inoculum	18-hr. culture	Subculture		
						1st	2nd	3rd
	(° C.)	(p.p.m.)						
Sterile skim, no phosphate	None	270	R-1	22×10^6	6×10^8	*	*	*
Sterile skim, 2% phosphate pH 6.55	None	157	R-1	22×10^6	19×10^7	*	*	*
Sterile skim, 2% phosphate pH 6.55	70–15 min.	56	R-1	22×10^6	25×10^6	43×10^5	28×10^6	*
Sterile skim, 2% phosphate pH 6.55	80–15 min.	58	R-1	22×10^6	18×10^4	1×10^4	120	0
Sterile skim, 2% phosphate pH 6.55	85–15 min.	48	R-1	22×10^6	138×10^4	21×10^4	110	0
Sterile skim, 2% phosphate pH 6.55	90–15 min.	27	R-1	22×10^6	105×10^4	6×10^3	40	0
Sterile skim, 2% phosphate pH 6.55	95–15 min.	25	R-1	22×10^6	95×10^4	2×10^4	20	0
Sterile skim, 2% phosphate pH 6.55	100–15 min.	21	R-1	22×10^6	9×10^5	7×10^3	50	0
Pasteurized skim, 2% phosphate pH 6.55	None	169	R-1	22×10^6	66×10^7	*	*	*
Pasteurized skim, 2% phosphate pH 6.55	100–15 min.	44	R-1	22×10^6	11×10^5	1×10^4	14×10^3	56×10^3
Pasteurized skim, 2% phosphate pH 6.55	100–30 min.	22	R-1	22×10^6	115×10^4	48×10^2	20	0
Pasteurized skim, 2% phosphate pH 6.55	100–60 min.	18	R-1	22×10^6	125×10^4	6×10^3	60	0
Pasteurized skim, 2% phosphate pH 6.55	100–90 min.	24	R-1	22×10^6	12×10^5	7×10^4	30	0

TABLE 3 (contd.)

Type of milk	Heat treatment	Calcium content	Lactic strain	Phage titer (No/ml)				
				Inoculum	18-hr. culture	Subculture		
						1st	2nd	3rd
	(° C.)	(p.p.m.)						
Pasteurized skim, 2% phosphate pH 7.28	None	157	ML-1	24×10^5	1×10^7	*	*	*
Pasteurized skim, 2% phosphate pH 7.28	100–15 min.	27	ML-1	24×10^5	21×10^4	14×10^4	37×10^3	56×10^4
Pasteurized skim, 2% phosphate pH 7.28	100–30 min.	18	ML-1	24×10^5	58×10^3	15×10^2	20	0
Pasteurized skim, 2% phosphate pH 7.28	100–60 min.	14	ML-1	24×10^5	31×10^3	6×10^2	30	0

* Culture lysed, no subculture.

The sterile and fresh milks were steamed for 15 and 30 min., respectively. Each milk was tested with Lactic Strain R-1 and its phage. Table 4 lists the

TABLE 4

Effect of milk solids on 2% phosphate treatment

Type of milk	Phosphated milk	Calcium content	R-1 phage titer (No/ml)			
			Inoculum	18-hr. culture	Subculture	
					1st	2nd
	(pH)	(p.p.m.)				
Fresh skim	6.52	35	22×10^6	77×10^4	1×10^4	20
Fresh reconstituted						
8% NFDM	6.60	10	22×10^6	61×10^4	16×10^3	0
10% NFDM	6.55	24	22×10^6	9×10^5	6×10^3	30
12% NFDM	6.52	29	22×10^6	20×10^5	15×10^3	70
14% NFDM	6.48	30	22×10^6	5×10^5	21×10^4	95×10^2
Sterile skim	6.36	31	22×10^6	86×10^4	6×10^3	50
Sterile reconstituted						
8% NFDM	6.42	15	22×10^6	83×10^4	48×10^2	20
10% NFDM	6.44	26	22×10^6	8×10^5	2×10^4	80
12% NFDM	6.40	37	22×10^6	25×10^5	18×10^5	28×10^3
14% NFDM	6.40	35	22×10^6	112×10^4	71×10^2	60

phage inoculum and the phage titers after one growth period and two subcultures in the respective media. The data with Strain R-1 indicate that the solids content of milk treated with phosphate was not too significant. However, tests with other lactic strains indicated that the phosphate treatment was more effective with low solids milk.

Effect of pyrophosphate. In an attempt to increase the effectiveness of the orthophosphate buffer in milk, varied quantities of pyrophosphate were tested in combination with orthophosphate. Reconstituted NFDM (10%) was divided into five lots and stock orthophosphate concentrate, pH 6.55, was added to yield 2.0, 1.9, 1.8, 1.7, and 1.6%, respectively. All lots were heated at 100° C. for 30 min. Then tetrapotassium pyrophosphate (50% concentrate) was added to yield 0.1, 0.2, 0.3, and 0.4% by weight in the lots of hot milk which already contained 1.9, 1.8, 1.7, and 1.6% orthophosphate, respectively. The fifth lot containing 2% orthophosphate served as a control.

The milks were cooled immediately and inoculated with *S. cremoris* ML-1 and its resistant phage. The phage titers at inoculation and after each of four subcultures in the respective media are presented in Table 5. All levels of pyrophosphate in combination with orthophosphate were more effective in suppressing bacteriophage proliferation than the 2% level of orthophosphate alone. Pyrophosphate at the 0.4% level had a marked reducing effect on the phage titer; no phage was detected after the first subculture.

TABLE 5

Effect of pyrophosphate as a supplement in orthophosphated milk

Potassium pyrophosphate	Orthophosphate buffer	Calcium content	ML-1 phage titer (No/ml)				
			Inoculum	18-hr. culture	Subculture 1st	Subculture 2nd	Subculture 3rd
(%)	*(%)*	*(p.p.m.)*					
None	2	24	450,000	80×10^6	32×10^7	*	*
0.1	1.9	20	450,000	12×10^4	14×10^4	175×10^3	30×10^5
0.2	1.8	13	450,000	47×10^3	15×10^3	7×10^3	200
0.3	1.7	17	450,000	80×10^3	4×10^3	0	0
0.4	1.6	19	450,000	13×10^3	0	0	0

* Culture lysed, no subculture.

Comparison of phosphate-treated milks. Thirteen strains of lactic bacteria, each having a different phage sensitivity pattern, and their homologous phages were tested in milks containing 2 and 3% orthophosphate buffer pH 6.63 and in milks containing 1.7% orthophosphate supplemented with 0.3% potassium pyrophosphate, to determine the number of transfers required to free each of phage. Three series of flasks containing pasteurized skimmilk forewarmed to 130° F. were treated with sufficient stock orthophosphate buffer pH 6.63 to yield 1.7, 2, and 3% by weight of the phosphate salt.

All flasks were heated at 100° C. for 30 min. Then, while still hot, the 1.7% orthophosphate was supplemented with 0.3% tetrapotassium pyrophosphate. All milks were cooled and one flask from each of the three phosphate treatments was inoculated with one of the lactic strains and its phage. Phage titers were determined after inoculation and after each of four serial subcultures in the same medium. They are presented in Table 6. All but two of the 13 different lactic strains were freed of phage with 2% orthophosphate. Results obtained with 3% orthophosphate were similar, except that the two resistant phages

TABLE 6

Effect of orthophosphate on different lactic phages

Lactic strain	Phage titer (No/ml)					
	Inoculum	18-hr. culture	Subculture			
			1st	2nd	3rd	4th
2% orthophosphate, pH 6.55						
R-1	25×10^6	49×10^4	32×10^2	10	0	0
US-3	1×10^8	28×10^4	9×10^2	10	0	0
ML-1	46×10^4	16×10^4	20×10^5	89×10^7	*	*
KH	23×10^6	17×10^4	160	10	0	0
HP	27×10^6	22×10^4	30	10	0	0
K	41×10^6	65×10^5	38×10^3	8×10^2	20	0
E-8	68×10^6	50×10^4	16×10^2	400	10	0
C-2	12×10^6	98×10^3	26×10^2	10	0	0
C-3	90×10^6	15×10^6	20×10^3	100	10	0
C-10	52×10^4	30×10^6	51×10^7	*	*	*
C-13	62×10^6	42×10^5	11×10^2	10	0	0
H-1	29×10^6	18×10^4	6×10^3	200	10	0
H-4	86×10^4	43×10^3	6×10^2	10	0	0
3% orthophosphate, pH 6.63						
ML-1	15×10^4	33×10^3	45×10^2	20	0	0
C-10	2×10^5	6×10^4	15×10^2	0	0	0

* Culture lysed, no subculture.

were eliminated from the lactic cultures. All 13 lactic types were freed of phage within two or three subcultures, in milk containing 1.7% orthophosphate supplemented with 0.3% pyrophosphate (Table 7).

TABLE 7

Effect of mixture of 1.7% orthophosphate and 0.3% pyrophosphate on different lactic phages

Lactic strain	Phage titer (No/ml)				
	Inoculum	18-hr. culture	Subculture		
			1st	2nd	3rd
R-1	125×10^5	58×10^3	21×10^2	0	0
US-1	20×10^6	40×10^3	20	0	0
ML-1	52×10^4	18×10^3	100	0	0
KH	90×10^5	27×10^4	60×10^3	50	0
HP	54×10^5	25×10^4	200	0	0
K	45×10^6	11×10^5	60×10^2	70	0
E-8	60×10^5	50×10^2	10	0	0
C-2	40×10^5	59×10^3	300	0	0
C-3	43×10^5	90×10^3	120	0	0
C-10	32×10^4	23×10^4	30×10^3	130	0
C-13	45×10^5	40×10^4	900	0	0
H-1	29×10^6	12×10^5	88×10^2	20	0
H-4	77×10^5	52×10^3	60	0	0

Effect of orthophosphate on activity of lactic cultures. The activities of single and mixed lactic cultures were determined after growth in skimmilks containing 2 and 3% orthophosphate.

Sterile, concentrated sodium and potassium phosphate buffers were prepared by mixing the phosphate salts in the ratios listed in Table 8, to yield final pH

TABLE 8

Effect of different orthophosphate salts on culture activity

Buffer combination						Titratable acidity (ml. N/20 NaOH)			
						Culture			
No.	Monobasic	Dibasic	Ratio	Phosphate	pH	W1	W2	W3	W4
				(%)					
....	None	—	......	....	......	10.4	11.3	12.1	11.5
1	$NaH_2PO_4 \cdot H_2O$	— Na_2HPO_4	3–1	2.0	6.24	11.1	12.4	13.4	12.2
2	$NaH_2PO_4 \cdot H_2O$	— Na_2HPO_4	2–1	2.0	6.52	9.9	9.8	10.4	9.9
3	$NaH_2PO_4 \cdot H_2O$	— Na_2HPO_4	1–1	2.0	6.8	7.0	8.2	6.8	7.1
4	$NaH_2PO_4 \cdot H_2O$	— K_2HPO_4	1–1	2.0	6.62	12.8	12.4	13.0	14.6
5	$NaH_2PO_4 \cdot H_2O$	— K_2HPO_4	1–1	3.0	6.62	10.2	12.1	12.9	12.0
6	$NaH_2PO_4 \cdot H_2O$	— K_2HPO_4	1–2	2.0	7.0	12.0	12.4	11.8	12.3
7	$NaH_2PO_4 \cdot H_2O$	— K_2HPO_4	1–4	2.0	7.28	11.1	12.8	11.0	13.4
8	KH_2PO_4	— Na_2HPO_4	3–2	2.0	6.57	11.0	11.1	12.4	12.6
9	KH_2PO_4	— Na_2HPO_4	3–2	3.0	6.57	8.9	11.4	10.5	9.0
10	KH_2PO_4	— Na_2HPO_4	1–1	2.0	6.82	8.8	10.6	10.1	8.2
11	KH_2PO_4	— K_2HPO_4	1–1	2.0	6.8	13.4	12.8	12.9	12.7
12	KH_2PO_4	— K_2HPO_4	1–1	3.0	6.8	10.7	11.5	12.7	12.0

* pH of 2% aqueous phosphate buffer.

values ranging from 6.24 to 7.28. The buffer solutions were added to sterile skimmilks in sufficient amounts to yield 2 and 3% of each salt. The phosphated milks were heated at 100° C. for 15 min., cooled, and inoculated with lactic culture. In most instances the lactic cultures were carried for two subcultures in the phosphated milk prior to determining their activity. Inoculation was at the rate of 1% and incubation was at 72° F. for 18 hr. The titratable activities of four representative commercial mixed lactic starters following growth in phosphated milks are listed in Table 8. Similar results were obtained with single strains.

The activity of several lactic cultures was reduced after they had been propagated for two transfers in phosphated milk containing only the sodium salt. The reduced activity was more striking as the pH of the buffer (No. 2, 3) was increased. However, the potassium salts (No. 11, 12), regardless of pH or concentration (2 to 3%), had no inhibitory effect on lactic cultures. Potassium and sodium phosphate buffer combinations (No. 4, 5, 6, 7, 8) had no inhibitory effect when equal ratios of sodium and potassium ions were present.

Effect of combination of ortho- and pyrophosphate on activity of lactic cultures. Single lactic strains and mixed commercial cultures were tested for acid production after at least two subcultures in milks containing varied percentages of both ortho- and pyrophosphate. Sufficient concentrated orthophosphate buffer, pH 6.63, was added to six lots of sterile skimmilk to yield 3, 2, 1.9, 1.8, 1.7, and 1.6% phosphate. Each lot of phosphated milk was steamed for 15 min. The portions with 2 and 3% orthophosphate received no further treatment. Pyrophosphate concentrate (50%) was added to the remaining four lots of milk in sufficient amounts to bring the total phosphate salt content to 2%. The reaction of the treated milks was approximately pH 6.5. The titratable acidities of four representative commercial mixed lactic cultures are presented in Table 9. In other studies, slight inhibition occurred with a few single strains

TABLE 9
Effect of tetrapotassium pyrophosphate on culture activity

Tetrapotassium pyrophosphate	Orthophosphate pH 6.6	Titratable acidity (ml. N/20 NaOH)			
		Culture			
		W1	W2	W3	W4
(%)	(%)				
None	None	13.2	12.5	12.8	10.1
None	2	15.0	13.1	14.7	14.5
None	3	10.0	12.3	11.0	10.4
0.1	1.9	12.7	12.6	12.7	12.9
0.2	1.8	10.3	12.3	12.5	10.1
0.3	1.7	9.8	9.6	12.7	11.8
0.4	1.6	9.0	11.5	12.6	12.4

when 0.4% pyrophosphate was added as a supplement; however, most single strains, like most mixed strains, remained as active as the controls without phosphate. To avoid slight inhibition of culture activity with some strains, 0.3% pyrophosphate should be used in combination with 1.7% orthophosphate.

DISCUSSION

In screening tests, various types of phosphates, including orthophosphate, metaphosphates, tripolyphosphates, and pyrophosphates were added to milk to determine their effect on phage proliferation. Orthophosphates were definitely more effective than any of the others and had less inhibitory effect on lactic culture activity. Pyrophosphates and tripolyphosphates tested individually were ineffective against phage, but they definitely improved the effectiveness of the orthophosphate treatment. However, they were only effective when added in the proper sequence, which was as follows: heating the milk, adding orthophosphate, steaming the mixture, and adding pyrophosphate or polyphosphate to the hot milk. Recently, Galesloot (6) showed that the lysis of a nisin-producing strain could be prevented by 1% polyphosphate alone. In our studies, polyphosphates alone had very little effect on most phage types. The simple addition of concentrated orthophosphate buffer, pH 6.6, to yield 2% phosphate by weight, followed by heating the milk at 100° C. for 30 to 45 min., was sufficient to prevent proliferation of most phage types and 3% prevented all types. However, 2% was sufficient to inhibit all types when the milk was buffered to pH 7.0.

Most of the orthophosphate concentrates containing different salt combinations could be added to milk in sufficient amount to provide 2% by weight of the salt without causing casein precipitation. However, some combinations did cause precipitation at the 3% level (for example, $K_3PO_4:NaH_2PO_4 \cdot H_2O$—ratio 3:7, pH 6.55).

All phosphate salts had to be added to the milk in the form of a liquid concentrate to prevent casein precipitation. Also, forewarming the milk to 130 to 150° F. and heating it immediately after the addition of phosphate was an essential procedure. Phosphate concentrates could be added aseptically to sterile milk without precipitation, provided the milk had been previously autoclaved at 120° C. for 15 min.

The addition of pyrophosphates to milks previously treated with orthophosphates did not appear to markedly reduce the content of unbound calcium, although its addition actively suppressed bacteriophage development. Therefore, it seems evident that the pyrophosphate plays another role in preventing phage proliferation in milk, probably by complexing other divalent metals.

In most instances, the activity of mixed- and single-strain lactic cultures grown in phosphated milk was equal to or greater than that of control cultures. No significant difference was found between the *Leuconostoc* content of mixed lactic cultures grown for four to six successive transfers in phosphated milk and that in the controls. Incubation periods less than 18 hr. at 72° F. did reduce the *Leuconostoc* content.

Kay (8) observed actual destruction of *Salmonella typhi* phage when exposed to solutions of phosphate buffer, citrate, and versene. In the present study, no loss in phage titers was observed when bacterial free phage filtrates were stored in phosphate-treated milks for 1 wk. Results of phage adsorption studies indicated that the phosphate added to milk greatly impaired adsorption of the phage to the host cell.

Results of this study offer two means for preventing the proliferation of phage in lactic bacterial starters, as follows:

1. To fresh or reconstituted skimmilk (9-14% solids), forewarmed to 130° F., add sufficient concentrated sodium-potassium orthophosphate (50%) buffer (Na_2HPO_4 and KH_2PO_4—Ratio 2:3), pH 6.5-6.6, to yield 1.7% of phosphate salt by weight, and steam the treated milk 30 to 45 min. While it is still hot, add sufficient concentrated solution (50%) of tetrapotassium pyrophosphate to yield 0.3% by weight of this salt. Cool and inoculate the phosphated milk in the usual manner and incubate for 18-20 hr. at 72-77° F.

2. To fresh or reconstituted skimmilk (9-12% solids) forewarmed to 130° F., add sufficient concentrated (50%) buffer (equal amount of K_2HPO_4 and $NaH_2PO_4 \cdot H_2O$, pH 6.6) to yield 3% phosphate salts by weight and steam the treated milk 30 to 45 min. Cool and inoculate the phosphated milk in the usual manner. Incubate for 18-20 hr. at 72-77° F.

The first procedure is preferred, because it is slightly more effective against phage proliferation, is less apt to cause milk precipitation, and reagents are more economical.

REFERENCES

(1) ADAMS, M. H. Bacteriophages. p. 235. Interscience Publishers, New York. 1959.

(2) ANONYMOUS. A Simple and Effective Method for Maintaining Bacteriophage-Free Lactic Starters. CA-E-19. EURDD, ARS, USDA. 1959.

(3) BABEL, F. J. New Developments in the Propagation of Lactic Cultures: Culture Media and Bacteriophage Inhibition. J. Dairy Sci., 41: 697. 1958.

(4) COLLINS, E. B., NELSON, F. E., AND PARMELEE, C. E. The Relation of Calcium and Other Constituents of a Defined Medium to Proliferation of Lactic Streptococcus Bacteriophage. J. Bacteriol., 60: 533. 1950.

(5) DOULL, DOROTHY P., AND MEANWELL, L. J. Phage Suppression and the Cultivation of Lactic Streptococci. Proc. 13th Intern. Dairy Congr., 3: 114. 1953.

(6) GALESLOOT, TH. E. The Propagation of Nisin-Producing Starters. Proc. 15th Intern. Dairy Congr., 2: 547. 1959.

(7) HARGROVE, R. E. A Simple Method for Eliminating and Controlling Bacteriophage in Lactic Starters. J. Dairy Sci., 42: 906. 1959.

(8) KAY, D. The Effect of Bivalent Metals on the Stability of a Typhoid Bacteriophage and Its Reaction with Its Receptor. Brit. J. Exptl. Pathol., 37: 560. 1956.

(9) MOSELEY, W. K., AND WINSLOW, R. L. A Study of Potential Causes of Culture Failures in Cheese Factories. J. Dairy Sci., 42: 906. 1959.

(10) POTTER, N. N., AND NELSON, F. E. Effects of Calcium on Proliferation of Lactic Streptococcus Bacteriophage. II. Studies of Optimum Concentrations in a Partially Defined Medium. J. Bacteriol., 64: 113. 1952.

(11) REITER, B. Inhibition of Lactic Streptococcus Bacteriophage. Dairy Ind., 21: 877. 1956.

(12) YALMAN, R. G., BRUEGEMANN, W., BAKER, P. T., AND GARN, S. M. Volumetric Determination of Calcium in Presence of Phosphate. Anal. Chem., 31: 1230. 1959.